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A new species of the *Drawida ghilarovi* species complex (Oligochaeta, Moniligastridae) in Changbai Mountain, Northeast China

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Abstract

The earthworm genus *Drawida* Michaelsen, 1900, has remained taxonomically and biogeographically unclear in China, with only one species described, *D. ganini* Zhang & Wu, 2021, which belongs to the *D. ghilarovi* Gates, 1969 species complex. Here, a new species, *D. yanbianensis* Liu & Zhao, **sp. nov.**, is described from Northeast China based on both morphological characters and molecular data. The new species is characterized by having a single pair of female pore slits on segment XII, a clitellum spanning five segments (X–XIV), a short body length (45.8–78 mm), having large and oval-shaped spermathecal ampulla with long coiled ducts, and having ovisacs from the ovary chamber on XI extending to XV. The new species status is supported with molecular data using the mitochondrial COI gene and four nuclear genes. The phylogenetic analysis shows that the new species is clustered with *D. ghilarovi* with a COI K2P genetic distance from the other species ranging from 12.4% to 20.9%. This study contributes to a deeper understanding of the evolution and distribution of the *D. ghilarovi* species complex and expands the known diversity within the genus *Drawida*.

Key Words

DNA barcoding, earthworm, Far East, Moniligastridae, taxonomy

Introduction

Earthworms are large soil animals that play an extremely important role in soil ecosystems, as well as in biological monitoring (Edwards 2004). However, the taxonomic work, particularly on the family Moniligastridae Claus, 1880, in contrast to other earthworm families, has been limited. The taxonomy of earthworms is somewhat limited by the simplicity of invertebrate structures, as they lack complex appendages or highly specialized copulatory organs (Pérez-Losada et al. 2009). Additionally, because earthworms are soft-bodied animals, their fossil record is extremely scarce (Piearce et al. 1990), making it challenging to trace their ancestors and evolutionary characteristics. The identification of earthworms primarily relies on the adult specimens with careful examination on the type of prostomium, arrangement of setae, position and morphology of the clitellum, male pores, spermathecal pores, and internal organs like testis sacs, spermathecae, ovisac, and gizzards. However, these morphological characteristics are variable, and different taxa may exhibit overlapping variability within the same character (Pop et al. 2003). Due to the limited availability of reliable taxonomic features, many morphologically similar

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species are either grouped as a single species with various morphological types or classified as species complexes. These complexes often encompass diverse taxonomic groups with uncertain taxonomic categories (Gates 1972; Sims and Gerard 1985; Briones 1993).

The earthworms in the family of Moniligastridae mainly occur in Asia, with possible origin in "east or near Myanmar" (Gates 1972, 1982; Jamieson 1977). Moniligastridae is essentially an Oriental family with some species distributed in the eastern Palearctic region (Stephenson 1923; Gates 1972). To date, Moniligastridae has 204 species belonging to five genera, with *Drawida* Michaelsen, 1900, being the most speciose with 147 species (Misirlioğlu et al. 2023). *Drawida* is widely distributed in India, Sri Lanka, Southeast Asia, China, the Korean Peninsula, Japan, and Far East Russia (Gates 1969; Blakemore et al. 2010; Narayanan et al. 2017, 2024; Zhang et al. 2020; Zhao et al. 2022).

Around 23 *Drawida* valid species and subspecies have been recorded in Northeast Asia (Zhang et al. 2021), with *D. ghilarovi* Gates, 1969, being the first species described in mountainous forests in Far East Russia (Gates 1969). The detailed description of the morph and distribution of *D. ghilarovi* was provided by Ganin et al. (2013, 2014), with three colorful morphs (black, brown, and grey) found among different individuals that occurred near the border of China and the Korean Peninsula.

Blakemore et al. (2014) firstly defined the *D. ghilarovi* species complex based on its wide distribution and similar regional congeners. *D. ghilarovi* shares certain similarities with several closely related regional congeners, such as *D. csuzdii* Blakemore, 2014; *D. guryeensis* Hong, 2002; *D. jeombongsan* Blakemore, 2014; and *D. tairaensis* Ohfuchi, 1938, with four gizzards in XIII–XVI, but these species exhibit distinct differences in morphological and genetic characteristics. The species complex includes six species: *D. csuzdii*, *D. ganini* Zhang & Wu, 2021, *D. ghilarovi*, *D. guryeensis*, *D. jeombongsan*, and *D. tairaensis*, and distributed in the Far East Russia, Northeast China, and the Korean Peninsula. In Northeast China, there is still a lack of taxonomic research on the morphological delimitation of the *D. ghilarovi* species complex. Existing studies have mainly focused on the analysis of the genetic differentiation of the complex, mainly based on mitochondrial data. Blakemore et al. (2010) first definitively associated a *Drawida* type specimen to its COI barcode. Atopkin and Ganin (2015) found significant genetic differentiation between two ecotypes of *D. ghilarovi* through the analysis of cytochrome c oxidase subunit I (COI) and 16S ribosomal RNA gene; Ganin and Atopkin (2018) first conducted the intraspecific molecular differentiation of *D. ghilarovi*, demonstrating that different ecotypes of *D. ghilarovi* possess distinct population structures; Zhang et al. (2021) described *D. ganini* based on the COI evidence and the pigmentation of black.

Shekhovtsov et al. (2022) revealed that *D. ghilarovi* in Far East Russia includes three genetic clades through the analysis of transcriptomic datasets. Clade I is only composed of grey morphs; clade II consists of both grey and brown morphs and contains the type location specimen of *D. ghilarovi* (GenBank accession HG970204); clade III not only contains the grey and brown morphs but also the black morphs of *D. ganini*. Shekhovtsov et al. (2022) concluded that pigmentation is not an important character for species delimitation of earthworms. However, except for the difference in pigmentation, *D. ganini* is distinguished from *D. ghilarovi* by having inconspicuous female pores, a smaller ovary chamber, a less coiled spermathecae duct, and the position of the clitellum in X–XV (vs. IX–XV) and the gizzard in XII–XV (vs. XIII–XVI). *D. ganini* in clade III should be a valid species, and the other lineages of clade III should be unsolved taxa.

A comprehensive collection of earthworms was done on the Changbai Mountains in Northeast China, where individuals of the *D. ghilarovi* species complex were observed to possess distinguishing morphological features. Here, we verify the phylogenetic relationship of members of the *D. ghilarovi* species complex using the mitochondrial and nuclear data with the establishment of a new species, *D. yanbianensis* Liu & Zhao sp. nov.

Materials and methods

Sampling

Earthworm specimens were collected during the summer of 2023, around the month of July, in Helong County (42.5717°N, 128.7817°E) and Antu County (42.5361°N, 128.2777°E), Yanbian Prefecture, Jilin Province, Northeast China. Sampling was done by digging and hand sorting. Collected samples were fixed in 100% ethanol for subsequent morphological and molecular analyses.

Morphological examination

External and internal characteristics of 15 clitellates (518R0_03, 518R0_05–10, 520R0_01–04, 520R0_06, and 520R0_08–10) were examined using a stereomicroscope (ZEISS) and ZEN 3.3. pro software for image capturing. Body length and width were measured; the features of the prostomium, the male and female pores, and the spermathecal pore, as well as the internal structures such as the spermathecae, testis sacs, and gizzards, were examined and documented.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from the posterior part of 34 earthworm specimens of *Drawida* using the TIANamp Genomic DNA Kit (Beijing, China) following the manufacturer's instructions. Fragment of the mitochondrial gene of the cytochrome c oxidase subunit I (COI) and two nuclear genes, 28S rRNA (28S) and

internal transcribed spacer 2 (ITS2), were amplified using the polymerase chain reaction (PCR). Two other nuclear genes, A-kinase anchor protein 17A (AKAP17) and flavin adenine dinucleotide synthetase 1 (FLAD1), were amplified using nested PCR. The primers used are shown in Table 1. The mixture (total volume 25 µl) contained 1 µl of template DNA and 17.25 µl of sterile ddH_2O, 2.0 µl of dNTPs, 2.5 µl of Easy Taq-Buffer, 0.25 µl of TransGen Easy Taq Polymerase, and 1 µl of each primer [forward and reverse primers, 10 uM]. The PCR protocol for COI was as follows: denaturation for 30 sec at 95 °C, annealing for 30 sec at 51 °C, and extension for 45 sec at 72 °C for 35 cycles with an initial denaturation step for 5 min at 95 °C and a final extension step for 5 min at 72 °C. For 28S and ITS2, the annealing temperature was 54 °C, while for AKAP17 and FLAD1, the annealing temperature was 48 °C. The PCR products were examined by electrophoresis in a 1% agarose gel and sent to Tianyi Huiyuan Biotechnology Co., Ltd. (Beijing, China) for sequencing using Sanger sequencing with an ABI 3730 automated sequencer. Sequences were aligned and edited using MEGA5 software (Tamura et al. 2011). All sequences and annotation information of the species were submitted to GenBank and shown in Table 2.

Molecular species delimitation analyses

Preliminary inference of species hypotheses through the distance matrix method of Assemble Species by Automatic Partitioning (ASAP) and the phylogenetic method of Generalized Mixed Yule Coalescent (GMYC) were applied for COI and 28S. ASAP is an automated species delimitation method based on COI and is designed to identify unique barcode gaps, that is, thresholds of genetic distance, thereby determining whether two individuals belong to the same species (Puillandre et al. 2021). ASAP is less prone to mismatches, has a short computation time, and is performed online (https://bioinfo.mnhn.fr/abi/public/asap).

GMYC is a species delimitation method based on speciation models (Fujisawa and Barraclough 2013) by fitting within and between species branching models to reconstruct gene trees. A bifurcated, rooted, ultrametric tree inferred by the BEAST package v1.7.5 (Drummond et al. 2012) and single-locus data as input files were provided to GMYC analysis performed in R 3.6.2 based on the splits v1.0–19 package.

Bayesian Phylogenetics and Phylogeography (BPP) is a Bayesian multispecies coalescent method that simulates explicitly the evolution of multi-gene data (Yang 2015; Flouri et al. 2018; Luo et al. 2018). BPP analyses were performed based on the five loci (COI, 28S, ITS2, AKAP17, and FLAD1) to confirm putative species. BPP v4.7 was used to estimate the species divergence times (τ) and population size parameters (θ) under the multispecies coalescent model on a fixed species phylogeny (A00). The population size parameters (θs) were assigned the inverse-gamma prior IG (3, 0.005), with a mean of 0.005/(3 – 1) = 0.0025. The divergence time at the root of the species tree (τ0) was assigned the IG prior (3, 0.005), with a mean of 0.0025, while the other divergence time parameters were specified by the uniform Dirichlet distribution (Yang and Rannala 2010). Species delimitation was performed using a fixed guide tree A10 in the within-model parameter posterior generated by running A00. The calculation of genetic distances using the Kimura-two-parameter (K2P) (Kimura 1980) model is shown in Table 3.

Phylogenetic analysis

Mitochondrial, nuclear, and the combined datasets were used to infer the phylogenetic relationship of the species in the *D. ghilarovi* complex, respectively. *Drawida cf. japonica* (Michaelsen, 1892) was set as outgroups. Maximum likelihood (ML) and Bayesian inference (BI) approaches were used to construct phylogenetic trees. ML analysis was performed in RAxML 8.0 (Stamatakis 2014) with the default rapid hill-climbing algorithm and the GTRGAMMA model to search for the best tree; the clade support value was

Table 1. Primers used for PCR and sequencing in this study.

Marker	Primer	Sequence (5'–3')	Round	Source
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG		Folmer et al. 1994
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA		
	COIE	TATACTTCTGGGTGTCCGAAGAATCA		Bely and Wray 2004
28S	28sF1	GAGTACGTGAAACCGTCTAG		Pérez-Losada et al. 2009
	28sR1	CGTTTCGTCCCCAAGGCCTC		
ITS2	E58S-F1	ATCACTGGGTTCGTGCGT		Shekhovtsov et al. 2013
	E28S-2	CCKCTTCACTCGCCGTTA		
AKAP17	AKAP17-F1	AAYTGGGARGTNATGGARAA	Round 1	This study
	AKAP17-R1	TCYTTRAACATNARYTTCAT		
	AKAP17-F2	AARATGATHAARCCNGAYCARTT	Round 2	
	AKAP17-R2	GCYTTNACRAANCCCATRTAYTC		
FLAD1	FLAD1-F1	GGNCCNACNCAYGAYGAYAT	Round 1	This study
	FLAD1-R1	TTNGGRTGNGTRTTYTCCAT		
	FLAD1-F2	TGYAARGCNTTYTTYGGNACNGA	Round 2	
	FLAD1-R2	TTNACNCKCATRAAYTCNGGCCA		

Table 2. List of *Drawida* species incorporated in the analysis.

Specimen ID	Species	Location	Latitude, Longitude	Accession Number				
				COI	28S	ITS2	AKAP17	FLAD1
518R0_01	*D. yanbianensis*	China: Jilin: Yanbian: Helong	42.5717°N, 128.7817°E	-	PQ415138	PQ415160	-	-
518R0_03	*D. yanbianensis*	China: Jilin: Yanbian: Helong	42.5717°N, 128.7817°E	-	PQ415140	PQ415168	-	-
518R0_04	*D. yanbianensis*	China: Jilin: Yanbian: Helong	42.5717°N, 128.7817°E	-	PQ415141	PQ415167	-	-
518R0_05	*D. yanbianensis*	China: Jilin: Yanbian: Helong	42.5717°N, 128.7817°E	-	PQ415142	PQ415165	-	-
518R0_06	*D. yanbianensis*	China: Jilin: Yanbian: Helong	42.5717°N, 128.7817°E	PQ411138	PQ415139	PQ415166	PQ423888	PQ427083
518R0_07	*D. yanbianensis*	China: Jilin: Yanbian: Helong	42.5717°N, 128.7817°E	PQ411139	PQ415134	PQ415164	PQ423889	PQ427082
518R0_08	*D. yanbianensis*	China: Jilin: Yanbian: Helong	42.5717°N, 128.7817°E	PQ411140	PQ415135	PQ415163	-	-
518R0_09	*D. yanbianensis*	China: Jilin: Yanbian: Helong	42.5717°N, 128.7817°E	PQ411141	PQ415136	PQ415162	-	-
518R0_10	*D. yanbianensis*	China: Jilin: Yanbian: Helong	42.5717°N, 128.7817°E	PQ411142	PQ415137	PQ415161	-	-
520R0_01	*D. yanbianensis*	China: Jilin: Yanbian: Antu	42.5361°N, 128.2777°E	PQ411143	PQ415147	PQ415159	-	-
520R0_02	*D. yanbianensis* (Paratype)	China: Jilin: Yanbian: Antu	42.5361°N, 128.2777°E	PQ411144	-	PQ415158	-	-
520R0_03	*D. yanbianensis*	China: Jilin: Yanbian: Antu	42.5361°N, 128.2777°E	PQ411145	-	PQ415157	-	-
520R0_04	*D. yanbianensis*	China: Jilin: Yanbian: Antu	42.5361°N, 128.2777°E	-	-	PQ415156	-	-
520R0_05	*D. yanbianensis*	China: Jilin: Yanbian: Antu	42.5361°N, 128.2777°E	PQ411146	-	-	-	-
520R0_06	*D. yanbianensis* (Holotype)	China: Jilin: Yanbian: Antu	42.5361°N, 128.2777°E	PQ411147	PQ415148	PQ415155	-	-
520R0_07	*D. yanbianensis*	China: Jilin: Yanbian: Antu	42.5361°N, 128.2777°E	PQ411148	PQ415144	PQ415154	PQ423891	PQ427084
520R0_08	*D. yanbianensis* (Paratype)	China: Jilin: Yanbian: Antu	42.5361°N, 128.2777°E	PQ411149	PQ415143	PQ415153	PQ423890	-
520R0_09	*D. yanbianensis*	China: Jilin: Yanbian: Antu	42.5361°N, 128.2777°E	PQ411150	PQ415145	PQ415152	-	-
520R0_10	*D. yanbianensis*	China: Jilin: Yanbian: Antu	42.5361°N, 128.2777°E	PQ411151	PQ415146	PQ415151	-	-
425DFH1	*D. ganini*	China: Heilongjiang: Muling River	46.049°N, 132.5939°E	-	PQ415125	PQ415171	-	-
425DFH2	*D. ganini*	China: Heilongjiang: Muling River	46.049°N, 132.5939°E	PQ411152	PQ415129	PQ415170	-	-
425DFH3	*D. ganini*	China: Heilongjiang: Muling River	46.049°N, 132.5939°E	PQ411153	PQ415130	PQ415172	-	-
425DFH4	*D. ganini*	China: Heilongjiang: Muling River	46.049°N, 132.5939°E	PQ411154	PQ415131	PQ415173	-	-
425DFH5	*D. ganini*	China: Heilongjiang: Muling River	46.049°N, 132.5939°E	PQ411155	PQ415132	PQ415169	PQ423895	PQ427088
425DFH6	*D. ganini*	China: Heilongjiang: Muling River	46.049°N, 132.5939°E	-	PQ415133	-	PQ423894	PQ427087
425DFH7	*D. ganini*	China: Heilongjiang: Muling River	46.049°N, 132.5939°E	PQ411156	PQ415126	PQ415174	-	-
425DFH8	*D. ganini*	China: Heilongjiang: Muling River	46.049°N, 132.5939°E	PQ411157	PQ415127	PQ415175	-	-
425DFH9	*D. ganini*	China: Heilongjiang: Muling River	46.049°N, 132.5939°E	-	PQ415128	PQ415176	-	-
425DFH10	*D. ganini*	China: Heilongjiang: Muling River	46.049°N, 132.5939°E	PQ411158	-	-	-	-
425DFH11	*D. ganini*	China: Heilongjiang: Muling River	46.049°N, 132.5939°E	PQ411159	-	-	-	-
425DFH12	*D. ganini*	China: Heilongjiang: Muling River	46.049°N, 132.5939°E	PQ411160	-	-	-	-
-	*D. ganini*	Russia: Jewish Autonomous Region(Bch): Bastak Reserve	48.5930°N, 135.0324°E	HG970192–HG970193	-	-	-	-
-	*D. ganini*	Russia: Khabarovsk Territory(Chch): Chirki River	48.0939°N, 135.0859°E	HG970194–HG970196	-	-	-	-
-	*D. ganini*	Russia: Khabarovsk Territory(Nch): Nanaian Region	49.2716°N, 136.4625°E	HG970197–HG970199	-	-	-	-

Specimen ID	Species	Location	Latitude, Longitude	Accession Number				
				COI	28S	ITS2	AKAP17	FLAD1
-	*D. ganini*	Russia: Primorye Territory(Pch): Razdol'naja River	43.3345°N, 131.5440°E	HG970200–HG970202	-	-	-	-
-	*D. ganini*	Russia: Primorye Territory(Khch): ike Khanka Reserv	44.38°N, 132.49°E	KY711475	-	-	-	-
-	*D. ganini*	Russia: Jewish Autonomous Region (Sch):v. Stolbowoe	47.54°N, 131.06°E	KY711476–KY711477	-	-	-	-
-	Group unsolved	Russia: Primorye Territory (SK): Sikhote-Alin Reserve	45.13°N, 136.29°E	KY711492	-	-	-	-
-	Group unsolved	Russia: Primorye Territory (Ss): Sikhote-Alin Reserve	44.59°N, 136.31°E	KY711493	-	-	-	-
-	Group unsolved	Russia: Primorye Territory (SsK): Sikhote-Alin Reserve	45.13°N, 136.29°E	KY711494–KY711495	-	-	-	-
-	Group unsolved	Russia: Southern Sikhote-Alin (ShK): Shivki Mountain	47.00°N, 134.22°E	KY711496–KY711498	-	-	-	-
-	Group unsolved	Russia: Kahabarovsk Territory: Southern Sikhote-Alin (ShS)	47.00°N, 134.22°E	KY711499–KY711501	-	-	-	-
-	Group unsolved	Russia: Primorye Territory (Lkd): Lazovsky Ridge	43.30°N, 133.35°E	KY711507	-	-	-	-
-	Group unsolved	Russia: Primorye Territory (Lkk): Lazovsky Ridge	43.30°N, 133.35°E	KY711508	-	-	-	-
-	Group unsolved	Russia: Primorye Territory (Vkd): Marine Biological Station "Vostok"	42.53°N, 132.44°E	KY711509–KY711510	-	-	-	-
-	Group unsolved	Russia: Primorye Territory (Vkk): Marine Biological Station "Vostok"	43.41°N, 132.09°E	KY711512–KY711514	-	-	-	-
-	*D. ghilarovi*	Russia: Primorye Territory: Kedrovaja Pad' Reserve (Ps)	42.2619°N, 130.3735°E	HG970204	-	-	-	-
-	*D. ghilarovi*	Russia: Primorye Territory (Uz): Ussurijskij Reserve	43.3325°N, 132.2118°E	HG970205	-	-	-	-
-	*D. ghilarovi*	Russia: Primorye Territory(GK): Biological Station "Gornotayozhnaya"	43.41°N, 132.09°E	KY711481	-	-	-	-
-	*D. ghilarovi*	Russia: Primorye Territory (Gs): Biological Station "Gornotayozhnaya"	43.41°N, 132.09°E	KY711483–KY711484	-	-	-	-
-	*D. ghilarovi*	Russia: Primorye Territory (Lk): Lazovsky Reserve	43.00°N, 133.44°E	KY711485–KY711487	-	-	-	-
-	*D. ghilarovi*	Russia: Primorye Territory(Uz): Ussurijskij Reserve	43.33°N, 132.21°E	KY711503–KY711506	-	-	-	-
-	*D. ghilarovi*	Russia: Primorye Territory (Ps): Kedrovaja Pad' Researve	42.26°N, 130.37°E	KY711516	-	-	-	-
-	Group6	Russia: Primorye Territory (Ps): Kedrovaja Pad' Researve	42.26°N, 130.37°E	KY711515	-	-	-	-
519R1_36	*D. ghilarovi*	China: Jilin: Yanbian: Wangqing	43.3257°N, 129.4149°E	PQ411161	PQ415150	-	-	-
519R1_38	*D. ghilarovi*	China: Jilin: Yanbian: Wangqing	43.3257°N, 129.4149°E	PQ411162	-	-	PQ423893	PQ427085
519R1_40	*D. ghilarovi*	China: Jilin: Yanbian: Wangqing	43.3257°N, 129.4149°E	PQ411163	PQ415149	-	PQ423892	PQ427086
R101	Group4	(Shekhovtsov et al. 2022)	-	OL785715	-	-	-	-
R123	Group unsolved	(Shekhovtsov et al. 2022)	-	OL785716	-	-	-	-

Specimen ID	Species	Location	Latitude, Longitude	Accession Number				
				COI	28S	ITS2	AKAP17	FLAD1
R108	Group6	(Shekhovtsov et al. 2022)	-	OL785717				
HN-LN-GR-I3_01	*D. cf. japonica*	China: Henan: Luoyang: Luoning	34.4364°N, 111.6368°E	PQ288577	PQ432447	-	PQ452810	PQ452826
HN-LN-GR-I3_02	*D. cf. japonica*	China: Henan: Luoyang: Luoning	34.4364°N, 111.6368°E	PQ288578	PQ432448	-	PQ452809	PQ452827
-	*D. angiang*	Vietnam: An Giang: Tinh Bien District	10.5882°N, 104.9506°E	ON303834	-	-	-	-
-	*D. cochinchina*	Vietnam: Dong Nai: Xuan Loc District	10.7931°N, 107.5257°E	ON303833	-	-	-	-
-	*D. cochinchina*	Vietnam: Dong Nai: Xuan Loc District	10.7931°N, 107.5257°E	ON303831	-	-	-	-
-	*D. gracilis*	(Ganin and Atopkin 2018)	-	JN887887	-	-	-	-
-	*D. gracilis*	(Ganin and Atopkin 2018)	-	JN793516	-	-	-	-
-	*D. bullata*	(Ganin and Atopkin 2018)	-	JN793527	-	-	-	-
-	*D. bullata*	(Ganin and Atopkin 2018)	-	JN887894	-	-	-	-
-	*D. nepalensis*	(Nguyen et al. 2022)	-	ON303830	-	-	-	-
-	*D. nepalensis*	(Nguyen et al. 2022)	-	MT472588	-	-	-	-
-	*D. japonica japonica*	(Nguyen et al. 2022)	-	EF077597	-	-	-	-
-	*D. gisti gisti*	(Nguyen et al. 2022)	-	JQ405262	-	-	-	-
-	*D. hattamimizu* (neotype)	Japan: Ishikawa: Kanazawa: Hatta	136.39°E, 36.34 °N	GQ500899	-	-	-	-
-	*D. hattamimizu*	Japan: Ishikawa: Kanazawa: Hatta	36.38°N, 136.41°E	AB543205	-	-	-	-
-	*D. nepalensis*	(Nguyen et al. 2022)	-	MH845467	-	-	-	-
-	*D. koreana*	(Nguyen et al. 2022)	-	KR047039	-	-	-	-
-	*D. koreana*	(Nguyen et al. 2022)	-	MH845538	-	-	-	-
IEW435-17	*D. ghatensis*	(Thakur 2021)	-	-	-	-	-	-
IEW434-17	*D. ghatensis*	(Thakur 2021)	-	-	-	-	-	-
IEW388-17	*D. brunnea*	(Thakur 2021)	-	-	-	-	-	-
IEW391-17	*D. impertusa*	(Thakur 2021)	-	-	-	-	-	-
IEW393-17	*D. impertusa*	(Thakur 2021)	-	-	-	-	-	-
IEW420-17	*D. circumpapillata*	(Thakur 2021)	-	-	-	-	-	-
IEW425-17	*D. travancorense*	(Thakur 2021)	-	-	-	-	-	-
IEW444-17	*D. robusta*	(Thakur 2021)	-	-	-	-	-	-
IEW445-17	*D. robusta*	(Thakur 2021)	-	-	-	-	-	-
IEW451-17	*D. scandens*	(Thakur 2021)	-	-	-	-	-	-
IEW459-17	*D. nilam*	(Thakur 2021)	-	-	-	-	-	-
HY-10	*D. jeombongsan*	(Blakemore et al. 2014)	-	-	-	-	-	-

Table 3. Percentage of K2P distances of COI of *D. yanbianensis* sp. nov. and the other species of the *D. ghilarovi* species complex. Values in parentheses showed intragroup distance (values in %).

	Species	1	2	3	4	5	6	7
1	Group1 (*D. yanbianensis*)	(0–9.1)						
2	Group2 (*D. ghilarovi*)	12.4–15.4	(0.2–7.1)					
3	Group3 (*D. jeombongsan*)	16.7–17.8	16.7–19.2	(0)				
4	Group4 (R101)	13.0–15.4	10.4–13.5	16.2	(0)			
5	*D. ganini*	16.9–20.9	16.3–20.3	19.2–21.2	16.9–19.0	(0–12.1)		
6	Group unsolved	16.9–19.4	13.6–18.8	18.1–20.7	14.1–17.0	8.1–16.2	(0.2–15.6)	
7	Group6 (R108)	17.1–18.8	15.6–19.9	17.4–18.5	16.5–19.5	16.5–21.0	16.7–21.3	(5.8)

assessed using 1000 rapid bootstrap replicates. BI was done in MrBayes v.3.2.6 (Ronquist et al. 2012) and ran in two million generations to ensure the average standard deviation of split frequencies is less than 0.01. The best nucleotide substitute models were chosen using jModelTest 2.1 (Darriba et al. 2012) based on the Akaike Information Criterion, which are TPM3uf+I+G for COI and SYM+I+G for the remaining three nuclear markers. The results of p files of BI were examined in Tracer v.1.7.2 (Rambaut et al. 2018), and the ESS larger than 200 were evaluated for convergence and to ensure sufficient burn-in for the trees. Both ML and BI trees were visualized and edited using FigTree v1.4.4 (Rambaut 2018).

Table 4. Comparison of key morphological characteristics of the *Drawida ghilarovi* species complex and related taxa (NA: not available).

Character	*D. yanbianensis*	*D. ganini*	*D. ghilarovi*	*D. csuzdii*	*D. guryeensis*	*D. jeombongsan*	*D. tairaensis*	*D. hattamimizu*	*D. nemora*	*D. ofunatoensis*
Color	Gray	Black	Brown or gray	Unpigmented in alcohol	Light pinkish or bluish	Light blue	Flesh coloured or pinkish and transparent	Dark blue-black	Dark blue	Yellowish
Length (mm)	45.8–78	66–121	100–142	120	62–83	60+	60–93	Averaging 246	65–185	228–283
Width (mm)	4.5–7.5	4.9–5.6	6	NA	2.3–2.5	NA	up to 2.7	9–10	Up to 6.5	Up to 6.5
Genital markings	6–12; irregular	6–10; irregular	5–13; irregular	Unilateral; irregular	8–10; paired	7/8/11/12; paired	Absent	6–9 or 10 or 11–12,13, paired	6–13; irregular	7–13; variously paired
Clitellum	10–14; greyish white	10–15; greyish white	9–15	$^{1}/_{2}$ 9–15; reddish	10–13; pinkish	Unclear	10–13	9/10–15/16; ash-grey	9/10–13/14; yellowish gray alive	10–13
Spermathecae	Ampulla large; duct long coiled	Ampulla medium; duct less coiled	Duct long coiled	Spherical ampulla; duct convoluted	Ampulla medium; duct long twisted	NA	Duct convoluted	Ampulla as sacs; duct long coiled	Duct convoluted	Duct convoluted
Spermathecal atrium	Absent	Absent	Absent	Absent	Present; 7/8	Absent	Present; 7/8	Absent	Absent	Absent
Ovisacs	11–15, palm shaped	11–12, palm shaped	11, long egg sacs	10/11/12, long, as far back as 14	12, extending to 16	NA	12–16	12 or 12/13/14, large paired	10/11/12 as far back as 14	11/12, short
Male pore	10/11; ventral bc lines, no penis	10/11; ventral bc lines, no penis	10/11; mediolateral in bc lines, no penis	10/11; mid-bc lines, no penis	10/11; two pairs, short and bla de-shape penis	10/11; no penis	10/11; no penis	10/11; ventral cd lines, no penis	10/11; penis small	10/11; small disc-shaped penis
Female pore	12; paired	Inconspicuous	11/12; paired	12 near 11/12; paired	11/12; paired	12; paired	12 near 11/12; paired	12; paired	12 near 11/12; paired	11/12; paired
Gizzard segments	Four; 12–17	Four; 12–15	Four; 13–16	Four; 13–16	Four; 13–16	Four; 13–16	Four; 13–19	Six; 13–18	Five; 12/13–15/16	Four; 12–17/18
Testis sacs	Ivory-white and large; IX–X(XI, XII)	IX–X	Hemispherical; IX–X	Spherical; IX–X	IX–X, mostly in X	X–XI	Spherical; X–XI (IX–X?)	IX–X	Spherical; IX–X	X–XI (IX–X?)
Vas deferens	Long coiled	Coiled	Long coiled	Slightly coiled	Long coiled	NA	Coiled	Long and convoluted	Slightly coiled	Coiled

Estimating divergence times

Divergence dates within the *D. ghilarovi* complex were estimated using a dataset of 74 COI sequences. Calibration was achieved by applying an earthworm mitochondrial COI clock rate of 0.024 substitutions per site per million years, as previously established by Chang and James (2011). The analysis was conducted using the BEAST v1.7.5 package, employing a strict clock model and a Yule speciation model. Two independent runs, each comprising 10,000,000 generations, were performed with sampling every 1,000 generations. The maximum clade credibility tree was subsequently generated using TreeAnnotator, summarizing node ages and posterior probabilities.

Abbreviations used in morphological figures

ag accessory gland;
amp ampulla;
fp female pore;
mp male pore;
sp spermathecal pore;
cl clitellum;
vd vas deferens;
ts testis sac;
gm genital markings;
os ovisac;
prg prostate gland;
p prostomium.

Results

Morphological characterization

Family Moniligastridae Claus, 1880

Genus *Drawida* Michaelsen, 1900

Generic diagnosis. Body size, small to giant [e.g., *D. japonica* (28~ mm); *D. hattamimizu* (~1000 mm)]. Prostomium prolobous. Clitellum is commonly in X–XIII, usually extending forward or backward [e.g., *D. nemora*

(IX, X–XIII, XIV)], or inconspicuous. Setae four pairs, lumbricine. Dorsal pores more usually absent or intermittently present [e.g., *D. companio* Blakemore, 2014 (15/16, 19/20/21, 25/26/27)]. Male pores in or near 10/11. Female pores usually paired in 11/12, or XII, or inconspicuous. Spermathecal pores paired in 7/8, or inconspicuous. A pair of testis sacs typically located in IX–X. A pair of ovisacs extends backward from ovary chamber (typically in XI). Spermathecae paired in VII–VIII. Two to six gizzards within segments XI–XVIII [or especially up to nine in *D. hattamimizu* extending to XX or XXI; five or six in *D. nilamburensis* Bourne, 1894 in XXVII–XXXIV]. Oesophageal gizzards, calciferous glands, and intestinal caeca absent.

Drawida yanbianensis Liu & Zhao, sp. nov.

https://zoobank.org/17775E03-10D2-4E7E-8003-274875BDBAFE

Fig. 4

Material examined. ***Holotype*:** • one clitellate (520R0_06) (COI accession number: PQ411147), Antu County (42.5361°N, 128.2777°E, 604 m elev.), Yanbian Prefecture, Jilin Province, 2023-07-21, coll. Huifeng Zhao. ***Paratypes***: • two clitellates [520R0_02 (COI accession number: PQ411144), 520R0_08 (COI accession number: PQ411149)], same data as of holotype.

Other specimens. 12 clitellates (518R0_03, 518R0_05 –10, 520R0_01, 520R0_03 –04, 520R0_09 –10), Helong County (42.5717°N, 128.7817°E, 42.6 m elev.), Yanbian Prefecture, Jilin Province, 2023-07-21, coll. Huifeng Zhao. All the specimens of the new species have been deposited in the Hebei Key Laboratory of Animal Diversity, Langfang Normal University, Hebei, China (C-HLU).

Etymology. The specific name of the species refers to the distribution area in Northeast China.

Diagnosis. Body grey, length 45.8–78.0 mm, diameter 2.3–2.6 mm, number of segments 91–144. Prostomium prolobous. Clitellum in X–XIV, ring-shaped. Setae: lumbricine, four pairs, existing in each segment. Spermathecal pores: one pair in 7/8, approximately on the setae cd line, ventro-laterally positioned, and spermathecal atrium absent. Dorsal pores absent. Genital markings: paired or unpaired, existing irregularly in segments VI–XII. Male pores: one pair, in intersegmental furrow 10/11, between setae b and c, near b, lip-shaped. Penis absent. Female pores: one pair slits on setae ab line of XII, near the 11/12 interval. Gizzards four in XII–XVII segments. Testis sacs: one pair, ivory-white present on IX–X, or elongate to XI or XII; vas deferens long coiled in IX–X, with one pair of elliptical prostate gland in X. Ovisac paired in XI–XV, palm-shaped. Four pairs of hearts in VI–IX. Spermathecae: one pair, hanging on the septum on 7/8, ampulla large in VIII, slightly yellowish, oval-shaped, with long coiled duct, spermathecal atrium absent.

Description. ***External characters*:** Length 45.8–78.0 mm, diameter 2.3–2.6 mm, 91–144 segments, body grey. Prostomium prolobous. Dorsal pores absent. Setae lumbricine, 4 pairs, small dots that exist in every segment except the first, about ab = cd, aa = 1.5 bc. Clitellum within X–XIV, greyish white and ring-shaped. Genital markings either paired or unpaired irregularly in VI–XII, or absent. Spermathecal pores, paired in 7/8, ventral, approximately on the setae cd line. Male pores, paired in 10/11, between setae b and c, near b, about 0.17 body circumference apart, lip-shaped, superficial without a copulatory organ, penis absent. Female pores, paired on setae ab line of XII, near the 11/12 interval.

***Internal characters*:** Hearts four pairs in VI–IX. Septa 7/8/9 relatively thick and muscular. Four gizzards in XII–XVII segments, milk-white. Testis sacs paired, ivory-white and large, each present on IX–X, or elongate to XI or XII, vas deferens long coiled in IX–X, with one pair elliptical prostate gland in X. Male atrium absent. Ovarian chamber complete, ovisacs paired, palm-shaped in XI–XV. Spermathecae paired in VII–VIII, ampulla large in VIII, slightly yellowish, oval-shaped, with long coiled duct, atrium absent. Accessory glands grape-like, stalkless, in correspondence with external genital markings.

Distribution. Helong County (42.5717°N, 128.7817°E) and Antu County (42.5361°N, 128.2777°E), Yanbian Prefecture, Jilin Province, Northeast China.

Habitat. Litter or subsoil geophage in forest soils.

Remarks. The new species is morphologically similar to *D. ghilarovi* and *D. ganini*, which are distributed in the Russian Far East and Northeast China (Gates 1969; Ganin et al. 2013; Zhang et al. 2021). They are similar in the position and number of male pores, spermathecal pores and spermathecae, prostomium, dorsal pores, and the absence of a penis. However, *D. yanbianensis* sp. nov. is distinguished from *D. ganini* and *D. ghilarovi* by having female pores on XII and the clitellum spanning five segments in X–XIV. The female pores of *D. ganini* are inconspicuous, while those of *D. ghilarovi* are located in the internode of 11/12; the position of the clitellum of *D. ganini* is located in X–XV, while that of *D. ghilarovi* is located in IX–XV. In addition, the new species (45.8–78 mm) is shorter than *D. ganini* (66–121 mm) and *D. ghilarovi* (100–142 mm) in body length. The new species have four gizzards in XII–XVII, but the position differs from that of *D. ganini*, which is in XII–XV, and that of *D. ghilarovi*, which is in XIII–XVI. The spermathecae of *D. yanbianensis* sp. nov. are oval-shaped with a large ampulla and a long coiled duct, while the ampulla of *D. ganini* is medium in size and has a less coiled duct.

The new species exhibits distinct morphological differences from the other species within the *D. ghilarovi* complex. *Drawida yanbianensis* sp. nov. lacks a penis in contrast to *D. nemora*, *D. guryeensis*, and *D. ofunatoensis* (Hong 2002; Blakemore et al. 2014). *Drawida hattamimizu* has six gizzards in XIII–XVIII, with an average length of 246 mm and a width ranging from 9 to 10 mm (Blakemore et al. 2010), which is much larger than that of the new species. Additionally, *D. yanbianensis* sp. nov. is approximately the same size as *D. csuzdii*, *D. jeombongsan*, and *D. tairaensis*, but the new species is gray in color with grayish-white clitellum, while *D. csuzdii* is unpigmented with reddish clitellum and *D. jeombongsan* is light blue with unclear clitellum (Blakemore et al. 2014). Moreover, the new species is characterized by its

grey coloration and a width ranging from 4.5 to 7.5 mm. In contrast, *D. tairaensis* exhibits flesh or pinkish coloration (Blakemore et al. 2014) and has a narrower (up to 2.7 mm) width than the new species.

Phylogenetic relationships and species delimitation

With the combined available data online, a total of 76 COI sequences revealed new phylogenetic relationships within the *D. ghilarovi* complex based on COI, with some individuals of our specimens scattered between clades II and III (Fig. 1). Based on the phylogenetic analysis and morphological characters, the *D. ghilarovi* complex is divided into seven groups. Different approaches support the identical topology of those groups, although the most support values are low (Fig. 1). The *D. ghilarovi* complex is shown to be a monophyletic group with a posterior probability (PP) of 100% and a bootstrap value (BV) of 71%.

The phylogenetic tree generated from the analysis of the multiple loci dataset, including COI, 28S, ITS2, AKAP17, and FLAD1, using both maximum likelihood (ML) and Bayesian inference (BI) methods (Fig. 2),

Drawida ganini Zhang & Wu, 2021
source from Zhang et al. 2021

Drawida yanbianensis **sp.nov.**
China: Jilin: Changbai Moutains

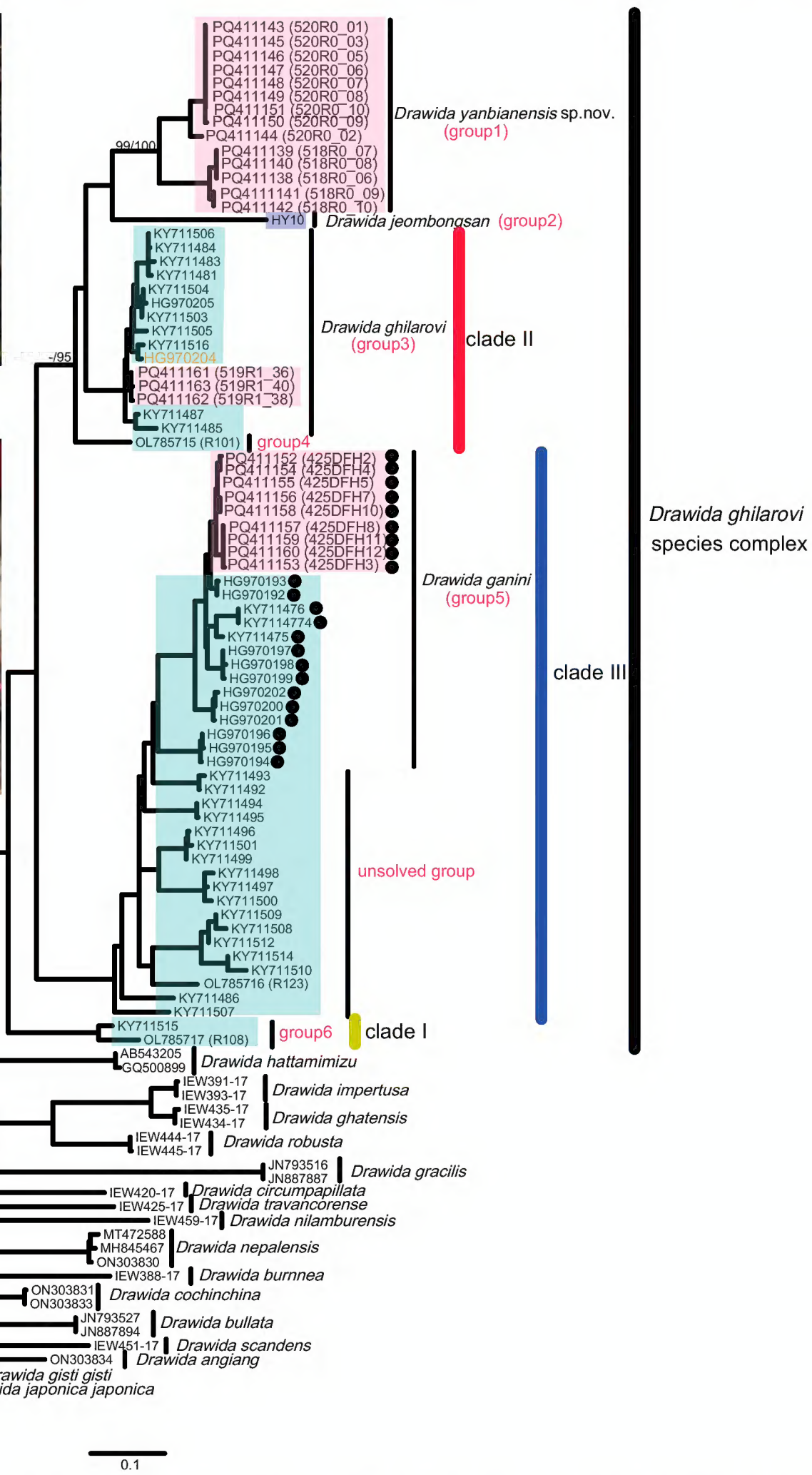

Figure 1. The phylogenetic tree based on COI using the maximum likelihood method. Numbers near branches indicate the maximum likelihood bootstrap support/Bayesian posterior probabilities. Posterior probability values lower than 80% and bootstrap values lower than 50% indicate weak support and are not shown.

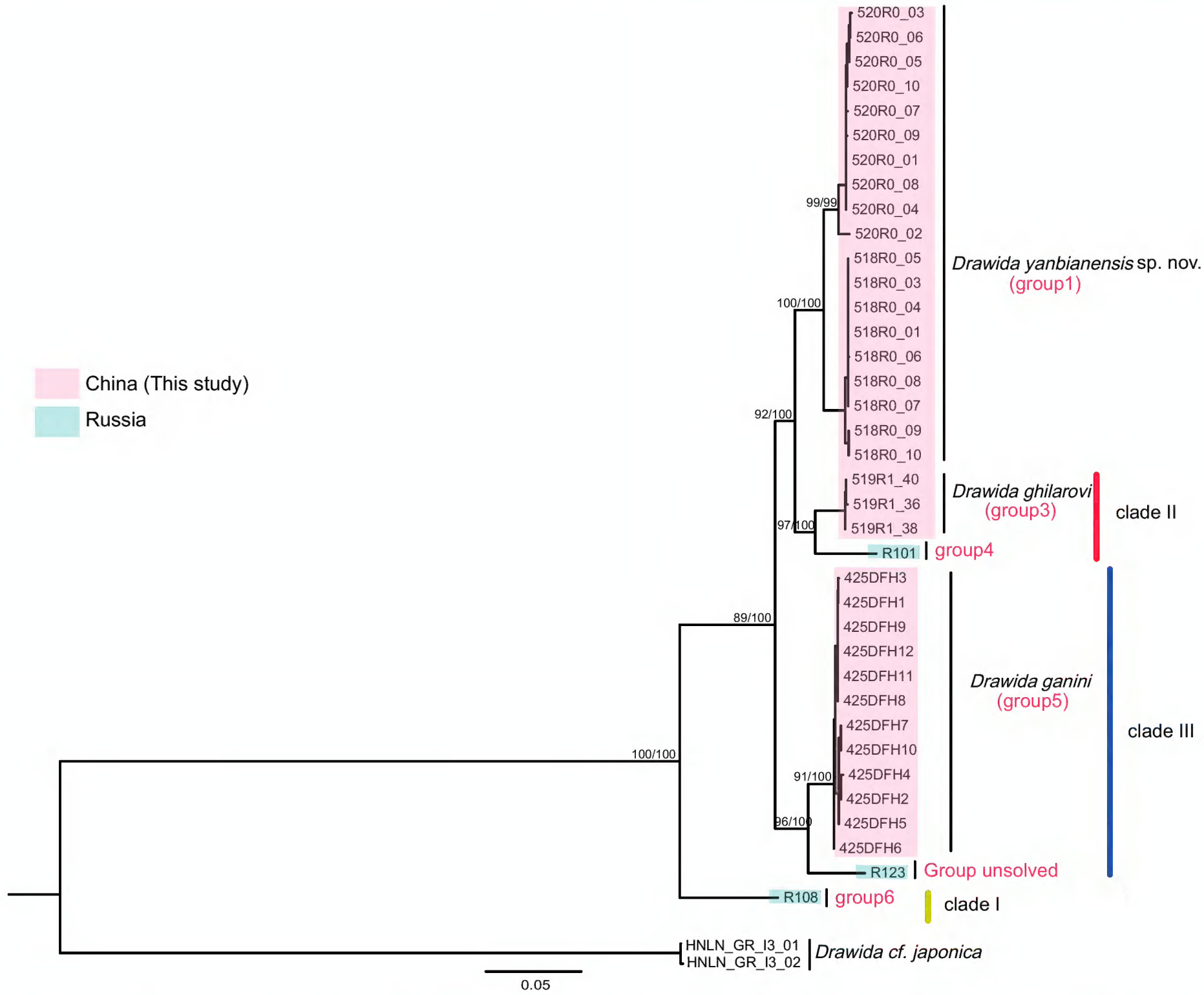

Figure 2. An ML concatenated tree of five genetic markers (COI, 28S, ITS2, FADL1, and AKAP17). Nodes with ML bootstraps > 70% are considered well-supported. Numbers near branches indicate the maximum likelihood bootstrap support/Bayesian posterior probabilities. The scale bar indicates the branch length.

exhibits a topological structure that is remarkably similar to the ML tree constructed solely with COI data (Fig. 1). In this tree, the clade corresponding to the *D. ghilarovi* complex is robustly supported, with both PP and BV being 100%. Additionally, the close relationship between the new species and *D. ghilarovi* is strongly corroborated, with a BV of 92% and a PP of 100%. In the concatenated phylogenetic tree of the *D. ghilarovi* complex (Fig. 2), the clades identified by Shekhovtsov et al. (2022) are as follows: Clade I, which comprises group 6, is strongly supported by high PP (100%) and BV (100%); Clade II, a well-supported clade with a BV of 97% and a PP of 100%, consists of members from groups 3 and 4; and Clade III, supported with a PP of 96% and a BV of 100%, is composed of members from groups 5 and 6.

The species delimitation by BPP into three Molecular Operational Taxonomic Units (MOTUs) is identical to the morphological classification (Fig. 3). ASAP also divides into three MOTUs using the 28S, consistent with BPP and morphological delimitation; however, it splits the new species into two MOTUs using COI. GMYC, on the other hand, delineates only one MOTU for all three species using 28S, while it splits the new species into two MOTUs and lumps *D. ganini* and *D. ghilarovi* using COI (Fig. 3). BPP is recognized as an accurate method for species delimitation because it incorporates multi-locus histories, which align with the multiple characters used in morphological approaches. In contrast, ASAP and GMYC consider only single-locus evolutionary histories. Here, ASAP demonstrated better performance compared to GMYC, a finding corroborated by another molecular species delimitation study on earthworms by Goulpeau et al. (2022).

Results of K2P analysis of COI show that the intraspecific sequence divergence for the new species ranged from 0–9.1% (Table 3, Suppl. material 1), and *D. yanbianensis* sp. nov. differs from the other species or group of *D. ghilarovi* complex ranging from 12.4%–20.9% (Table 3). The ancestor of the *D. ghilarovi* complex emerged around 9.0 million years ago (Ma; 95% credible range of 11.2–7.2 Ma) during the Late Miocene (Suppl. material 2). Most groups within the *D. ghilarovi* complex diverged between approximately 8.2 and 3.7 Ma. The two subclades of *D. yanbianensis* sp. nov. split around 2.8 Ma, coinciding with the onset of the Quaternary Period (Suppl. material 2).

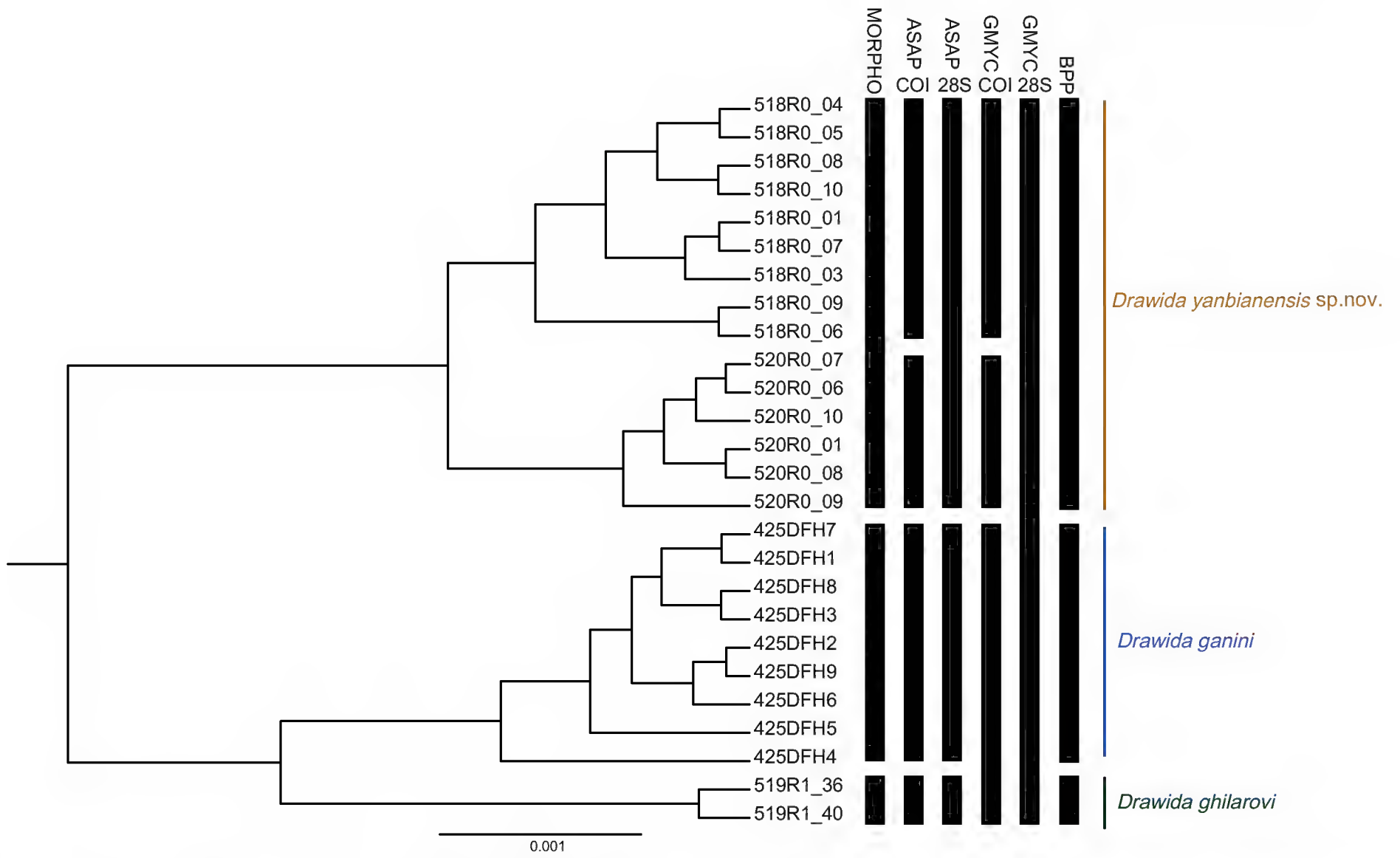

Figure 3. Species delimitation of the *Drawida ghilarovi* species complex. ASAP, automated barcode gap discovery; GMYC, generalized mixed Yule coalescent model; BPP, Bayesian phylogenetics and phylogeography.

Discussion

The earthworms of Moniligastridae mainly occur in Asia, with their probable origin inferred to be in the 'east or near Myanmar' (Gates 1972, 1982; Jamieson 1977; Narayanan et al. 2024). This inference is based on the fact that four out of the five known genera of Moniligastridae are naturally distributed in the eastern or nearby areas of Myanmar. The widespread distribution of Moniligastridae is largely attributed to the dispersal of *Drawida* species. *Drawida* was described by Stephenson (1923: 118, 124) as "one of the large Indian genera"; its headquarters appear to be in Sri Lanka, southern India, eastern Himalayas, and Myanmar (Blakemore 2009). The historical migration patterns of *Drawida* across Asia, especially East Asia, where the *D. ghilarovi* complex occurs, are unknown until Gates (1972). A more comprehensive sampling strategy that covers the entire distribution area of the genus for elucidating the phylogeographic patterns and evolutionary history of *Drawida* is essential in the future.

Temperature and food resources are the primary variables restricting the growth and productivity of earthworms (Fayolle et al. 1997). The mountains in northeastern Asia are well-preserved alpine ecosystems in high-altitude regions (Yang and Xu 2003). Earthworms inhabiting these areas are exposed to significant temperature fluctuations and are cold-tolerant, such as the members of the *D. ghilarovi* complex (−16 °C in worms to −20 °C for cocoons) (Berman et al. 2010), and many lumbricid species, such as *Eisenia nordenskioldi* (Eisen, 1879), *Aporrectodea tuberculata* (Eisen, 1874), *A. trapezoides* (Dugès, 1828), *etc*., also occur in this region (Shekhovtsov et al. 2018, 2020, 2023; Zhao et al. 2022). Pheretimoid is the advantaged earthworm group in most habitats around the world, but they are intolerant to the cold environment as the cocoons are killed below 5 °C (Richardson et al. 2009; Görres et al. 2018). However, few *Pheretimoid* species are found in the alpine forests of East Asia, with only one exception thus far (Dong et al. 2024), presumably due to their intolerance of cold habitats. Therefore, the *D. ghilarovi* complex and some lumbricid species share the cold and fertilized mountain niche. The gut microbiota may provide multiple physiological benefits to their hosts, especially in adapting to temperature and food resources (Yang et al. 2023). A study by Hoek et al. (2016) showed that the cooperation of gut bacterial communities can enhance the tolerance of *E. nordenskioldi* to harsh environmental conditions in high-latitude cold regions. Thus, with the advancement of earthworm genomics research, especially studies based on whole genomes, the mechanisms of cold tolerance in alpine earthworms will be further elucidated.

The geographic isolation of the mountainous region in Northeast Asia is a primary driver of speciation within the *D. ghilarovi* complex. Through molecular and morphological analyses, the delimitation of the *D. ghilarovi* complex has been performed, with the description of a new endemic species likely resulting from allopatric speciation. In this study, two distinct lineages identified within the species *D. yanbianensis* sp. nov. in Helong (518R0) and Antu (520R0) counties exhibit a significant population genetic divergence, as evidenced by a 9.1% K2P distance.

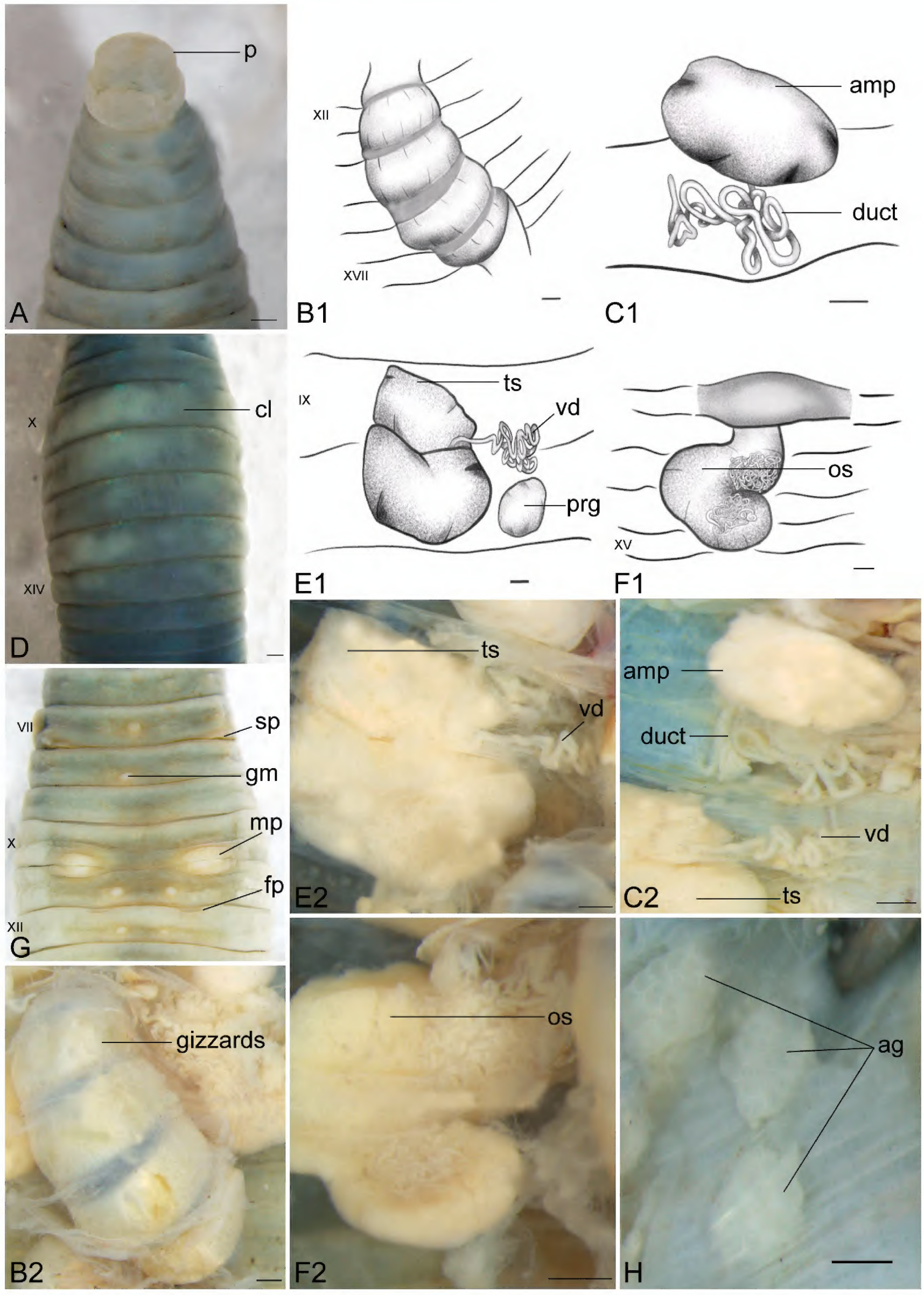

Figure 4. *Drawida yanbianensis* sp. nov., holotype (520R0_06). **A.** Ventral view of the prostomium; **B1, B2.** Ventral view of gizzards; **C1, C2.** Left spermathecae; **D.** Dorsal view of clitellum region; **E1, E2.** Left testis sac and prostate gland; **F1, F2.** Left ovisac; **G.** Ventral view of genital markings and the spermathecal pores, male pores, female pores; **H.** Ventral view of right accessory glands. Scale bars: 0.5 mm.

This genetic differentiation suggests that these populations are likely undergoing the process of allopatric speciation (Shekhovtsov et al. 2022). An in-depth study of this complex in the future can help us better understand the evolutionary history of montane species in Northeast Asia.

It is widely acknowledged that the *D. ghilarovi* complex exhibits morphological conservatism, as referred to in the review by Narayanan et al. (2024). Consequently, the utilization of molecular data is deemed essential for the accurate delimitation of species within this complex.

The findings of this study indicate that the COI gene alone may potentially lead to an overestimation of species boundaries. In contrast, a multi-locus coalescence-based species delimitation approach, particularly when incorporating nuclear markers, demonstrates a significantly enhanced accuracy in the assignment and delineation of species (Fig. 3). This study exhibits additional potential species diversity in the *D. ghilarovi* complex in Northeast Asia, exemplified by the unresolved group as depicted in Fig. 1. This underscores the necessity for further taxonomic scrutiny of the complex. The subsequent step should involve integrative taxonomic analyses encompassing morphological, genetic, ecological, and behavioral data for the *D. ghilarovi* complex. Additionally, current phylogenetic and phylogeographic studies of the *D. ghilarovi* complex are notably deficient in molecular data from related species such as *D. csuzdii*, *D. guryeensis*, and *D. tairaensis*. This gap highlights the imperative for future research to address this shortfall and provide a more comprehensive understanding of the complex.

Conclusion

In this study, an integrative taxonomic approach, incorporating both morphological and molecular data, has been employed to delimit a new species, *D. yanbianensis* sp. nov., within the *D. ghilarovi* complex. This delimitation approach significantly augments taxonomic precision, thereby holding immense significance for the field of earthworm taxonomy. Furthermore, it contributes to the enrichment of the biodiversity of the Changbai Mountains region in Northeast China. This discovery is instrumental in deepening our understanding of the complexity and stability of the local ecosystem and offers valuable data and research opportunities for the conservation of biodiversity.

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Supplementary material 1

The intraspecific K2P distances of COI of *D. yanbianensis* sp. nov.

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Data type: docx

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Supplementary material 2

The time framework of the *Drawida ghilarovi* species complex was calibrated using a nucleotide substitution rate of 2.4% per site per million years for the COI gene

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Data type: ai

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